



METABOLIC, ENDOCRINE, AND GENITOURINARY PATHOBIOLOGY

Age-Related Alterations in Regeneration of the Urinary Bladder after Subtotal Cystectomy

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Prior work documented that surgical removal of approximately 70% of the bladder (subtotal cystectomy) in 12-week-old female rats induced complete functional regeneration of the bladder within 8 weeks. To determine whether animal age affects bladder regeneration, female F344 rats aged 12 weeks (young) and 12 months (old) underwent subtotal cystectomy, and then were evaluated from 1 to 26 weeks after subtotal cystectomy. At 26 weeks after subtotal cystectomy, bladder capacity in young animals was indistinguishable from that in age-matched controls, but bladder capacity in old animals was only approximately 56% of that in age-matched controls. There was no detectable difference in residual volume among treatment groups, but the diminished regeneration in old animals was associated with a corresponding increase in the ratio of residual volume to micturition volume. The majority of old animals exhibited evidence of chronic kidney damage after subtotal cystectomy. Maximal contraction of bladder strips to electrical field stimulation, as well as activation with carbachol, phenylephrine, and KCl, were lower in old than in young animals at 26 weeks after subtotal cystectomy. Immunostaining with proliferating cell nuclear antigen and Von Willebrand factor revealed delayed and/or diminished proliferative and angiogenic responses, respectively, in old animals. These results confirm prior work and suggest that multiple mechanisms may contribute to an age-related decline in the regenerative capacity of the bladder. (*Am J Pathol* 2013, 183: 1585–1595; <http://dx.doi.org/10.1016/j.ajpath.2013.07.018>)

Animal experiments have demonstrated that the bladder possesses a remarkable regenerative capacity. Despite conflicting reports, there also are numerous indications that the human bladder can regenerate after removal of a large portion of the organ in a subtotal cystectomy (STC).^{1–8} The potential utility of intrinsic bladder regeneration for the development of novel clinical therapies is exemplified by the work of Atala et al,⁹ who successfully implanted engineered autologous neobladders into patients with myelomeningocele.

Although the regenerative powers of the mammalian bladder have been known for decades, the overwhelming majority of animal studies have examined regeneration after implantation of scaffolds with or without cells for bladder augmentation. Few studies have characterized *de novo* bladder regeneration after trigone-sparing STC alone (ie, in the absence of any exogenous intervention). To put this in proper perspective, recent studies have demonstrated complete functional rodent bladder regeneration after STC, with

surgical removal of 70% to 80% of the bladder.¹⁰ That regenerative response is a very different phenomenon from the process studied in the bladder augmentation models commonly used to evaluate the effects of various stem cells and tissue-engineering biomaterials on bladder regrowth. Frederiksen et al^{11,12} conducted pharmacological and morphological analyses after STC in female rats and found that the newly formed detrusor smooth muscle was similar to the trigone from which it had developed with respect to both pharmacological properties and pattern of innervation. Saito et al¹³ found that bladder capacity returned to approximately 59% of control values 4 weeks after STC in male rats, and Piechota et al¹⁴ observed normal bladder

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capacities 4 months after STC, in both male and female rats (3 months old). Burmeister et al¹⁰ also described functional regeneration of the bladder after trigone-sparing cystectomy (STC) in 12-week-old female Fisher F344 rats.¹⁰ In their studies, the bladder had regrown to a normal size by 8 weeks after STC as determined by both computed tomographic imaging and *in vivo* urodynamic analysis. Moreover, there was sufficient recovery in contractile strength of detrusor smooth muscle to ensure normal bladder emptying during micturition. Morphologically, the bladder had all three layers (urothelial, lamina propria, and detrusor muscle), and wall thickness was normal by histological evaluation.

A more recent follow-up study from our research group used fluorescent bromodeoxyuridine labeling to quantify the spatiotemporal characteristics of the proliferative response that accompanies functional regeneration during the critical first week after STC.¹⁵ Fewer than 1% of cells in the bladder wall were labeled with bromodeoxyuridine in control bladders under resting conditions (ie, no damage), but this percentage increased, by fivefold to eightfold, at all time points after STC. Such observations clearly document that the early stages of functional bladder regeneration are characterized by time-dependent changes in the location of the proliferating cell population in all three bladder wall layers, and furthermore demonstrate time-dependent expression of several evolutionarily conserved developmental signaling proteins (ie, sonic hedgehog protein, *gli1*, and bone morphogenic protein-4) during this same time frame.¹⁵ As far as we are aware, bladder regeneration is unique with respect to its regenerative potential, and there is no other mammalian organ capable of this type of anatomical and functional regeneration. The present study extends our previous observations on 12-week-old rats to document a significant diminution in regenerative capacity in rats that are 12 months old. These findings, and the putative mechanisms responsible, may have important implications for the development of novel therapeutics for the treatment of diverse bladder diseases and disorders.

Materials and Methods

Animals

A total of 45 young (defined as 12 weeks of age) and 103 old (defined as 12 months of age) female Fisher F344 rats were used in this study. The young rats weighed 170 to 200 g, and the old rats weighed 250 to 300 g. Of the young animals, 38 underwent STC; in 15 of these animals, bladders were excised for histology (three at each of 1, 2, 4, 8, and 26 weeks after STC), and the remaining 23 STC animals were assigned to urodynamic or pharmacological studies at 26 weeks. Of the old animals, 82 underwent STC; in 15 of these animals, bladders were excised for histology (three each at the same five time points as for young animals), and the remaining 67 animals were used for urodynamic and pharmacological experiments performed at 2, 4, 8, and 26 weeks after STC.

The age-matched controls (AMC; 7 young and 21 old animals) did not undergo STC. All protocols were approved by the Institutional Animal Care and Use Committee of Wake Forest University.

Surgical Procedures

Trigone-Sparing Cystectomies

Rats were anesthetized with 2% isoflurane. Animals were anesthetized with isoflurane, and the abdominal wall was shaved. Povidone-iodine was used as an antiseptic to decontaminate the surgical site. A low midline abdominal incision was made, and the bladder dome was dissected down to the level of ureteral insertion. Two stay sutures were made on either side of the bladder, just above the ureteric orifices, using 6-0 polyglycolic acid sutures. The dome portion of the bladder (60% to 70%) was excised, leaving the trigone and ureterovesical junctions intact (trigone-sparing cystectomy). The remaining portion of the bladder was then sutured in a continuous fashion using one of the original stay sutures. The abdominal wall and skin were closed in two layers using 3-0 Vicryl sutures. Animals were allowed to recover and were given food and water *ad libitum* for up to 6 months after STC.

Bladder Catheter Implantation

Rats were anesthetized with 2% isoflurane. Through a midline incision, the bladder was dissected from adhesions due to the previous surgery. The bladder dome was delivered outside of the body as described above, and a small incision was made. A PE-50 Intramedic polyethylene catheter (Becton Dickinson, Franklin Lakes, NJ) with cuff was inserted and anchored with a 5-0 purse-string silk suture. The catheter was then tunneled subcutaneously and brought out through the animal's nape and was held in place with cloth tape anchored to the skin via a 3-0 Vicryl suture. The abdominal wall and skin were closed in two layers with 3-0 V Vicryl sutures, and the free end of the catheter was thermally sealed.

Cystometric Analysis

All cystometric studies were performed 3 days after catheter implantation in conscious, freely moving rats. In brief, the previously implanted bladder catheter was connected to a three-way valve that was, in turn, connected to a pressure transducer and an infusion pump. The pressure transducer was connected to a transducer amplifier (ETH 400; CD Sciences, Dover, NH), which was, in turn, connected to a data acquisition board (MacLab/8e; Analog Digital Instruments, Castle Hill, NSW, Australia). The pressure transducers and acquisition board were calibrated in cmH₂O before each experiment. Saline solution at room temperature was infused at a rate of 10 mL/hour. Micturition volumes were measured with a silicone-coated funnel leading into a collection tube connected to a force-displacement transducer. Analysis was begun after a consistent voiding pattern was established. The following cystometric parameters were

investigated: basal pressure (BP), the lowest pressure between voids; maximum pressure (MP), the highest bladder pressure during micturition; threshold pressure (TP), the pressure that initiates a voiding contraction; bladder capacity (BC), the residual volume after the last micturition plus the amount of saline infused; micturition volume (MV), the amount of expelled urine; residual volume (RV), the bladder capacity less the micturition volume; and bladder compliance (B_{com}), calculated as $BC/(TP - BP)$.

Preliminary statistical analysis revealed that urodynamic parameter estimates for the old AMC animals at 2, 4, and 8 weeks after STC were indistinguishable; these animals were therefore considered to be homogeneous and were treated as one group (pooled as controls).

Renal Function

Animals were placed in individual cages on an 18-cage metabolic rack (Allentown, Inc., Allentown, NJ) for separation of urine from feces and food debris. Urine collection began at the start of the diurnal dark cycle and continued for 20 hours. Before and after urine collection, weights were recorded for all animals, water bottles, and urine tubes. After collection, urine was centrifuged at $1300 \times g$ for 15 minutes to remove any particulate contamination, and the supernatant was separated into 1-mL aliquots and immediately stored at -80°C . Upon euthanasia, at least 2 mL of blood was collected from the heart and placed in a serum-separating VacuTainer tube (367981; BD Biosciences, San Jose, CA). Tubes were spun at $2000 \times g$ for 15 minutes at 4°C to separate serum. Supernatant (serum) was removed, separated into 250- μL aliquots, and immediately stored at -80°C . Creatinine and blood urea nitrogen analysis were performed with a Synchron CX5 chemistry analyzer (Beckman Coulter, Brea, CA), with output units of serum and urine creatinine as mg/dL. Creatinine (CR) clearance was calculated as $\text{Clearance} = [\text{CR}_{\text{urine}} \times \text{Volume}_{\text{urine}} (\text{mL})] / [\text{CR}_{\text{serum}} \times \text{Time} (\text{min})]$ and was normalized per gram body weight.

Pharmacological Studies

Animals were sacrificed with CO_2 inhalation and bilateral thoracotomy. The bladders were harvested and immediately placed in ice-cold Krebs buffer. The bladders were cut into approximately equally sized strips along the longitudinal axis. The strips were denuded of the urothelium and suburothelium and then were attached to tissue holds at one end and to force transducers at the other end in an organ bath system (Danish Myo Technology, Aarhus, Denmark) containing 15 mL of Krebs buffer aerated with 95% O_2 /5% CO_2 at 37°C . Bladder strips were subjected to a resting tension between 1 and 1.5 g and were allowed to stabilize for at least 60 minutes. Strips were then primed with 60 mmol/L KCl-induced contractions (recorded as changes in tension from baseline) until subsequent contractions were consistent. Cumulative, steady-state carbachol concentration response

curves (CRCs) were generated by stimulating muscarinic receptors with increasing concentrations of carbachol at half-log increments ranging from 3 nmol/L to 100 $\mu\text{mol/L}$. Phenylephrine CRCs were generated by stimulating α_1 adrenoreceptors with increasing concentrations of phenylephrine in full-log increments ranging from 10 nmol/L–100 $\mu\text{mol/L}$. For the electrical field stimulation protocol, bladder strips were placed between two platinum electrodes in the organ chamber, and electrical pulses (0.1 ms pulse width, 20V in the bath) were delivered, lasting 30 seconds each at increasing frequencies (1, 2, 4, 8, 16, and 32 Hz) using an S88 stimulator (Grass Instrument, West Warwick, RI). All contractile responses were normalized to grams of tissue weight.

Histological Analysis

Bladders not subjected to pharmacological analysis were preserved for histology. Bladders were fixed in 10% buffered formalin overnight, processed, embedded in paraffin, and cut into 7- μm axial slices. All staining was performed on at least two different areas of the bladder. The first cross section was taken closer to the most distal edge of the bladder (approximately 4 mm from the bladder dome), and another was taken from a more proximal location (approximately 4 mm distal from the ureterovesical junction). Slides were cleared in xylene and rehydrated to water, where they were ready for staining.

Gomori's trichrome staining (9176A; Newcomer Supply, Middleton, WI) was performed, and IHC was performed as follows. A heat-mediated antigen retrieval step was used with 0.01 mol/L citrate buffer at 95°C for 15 minutes. Endogenous peroxidase activity was blocked with dual endogenous enzyme-blocking reagent (S2003; Dako, Carpinteria, CA) for 10 minutes at room temperature, followed by 10 minutes of incubation with serum-free protein block (X0909; Dako). Samples were then incubated with a primary antibody to Von Willebrand factor (VWF) (rabbit polyclonal, 1:200 dilution; A0082; Dako) for 60 minutes. After primary antibody incubation, slides were treated for 30 minutes each with biotinylated universal link and streptavidin horseradish peroxidase (K0690; Dako). Finally, staining was completed with 5 to 10 minutes of incubation with ImmPACT diaminobenzidine (SK-4105; Vector Laboratories, Burlingame, CA). IHC probing proliferating cell nuclear antigen (PCNA) was performed similarly, but without blocking endogenous peroxidase activity and without using diaminobenzidine as a chromogen. The primary antibody used was against PCNA (mouse monoclonal, 1:3000 dilution; ab29; Abcam, Cambridge, MA). The secondary antibody was conjugated with Texas Red (1:250 dilution; TI-2000; Vector Laboratories).

Image Analysis

Image analysis was performed with Image-Pro software version 6.3 (Media Cybernetics, Bethesda, MD). One proximal and one distal section were taken for each animal

designated for histology; four high-magnification images were taken in each section. For trichrome analysis, the color selection tool was used to determine the quantity of pink (muscle) and blue (collagen) pixels; the percentage of muscle corresponds to the number of pink pixels divided by the total number of selected pixels. For PCNA analysis, sections were counterstained with DAPI, and PCNA-positive nuclei were counted and divided by the total number of nuclei. For vessel quantification, images were spatially calibrated, and the inner lumen was traced with the polygon measurement tool. Native tissue taken out at the time of STC in old and young animals was used for controls.

Statistical Analysis

Statistical evaluations were performed using GraphPad Prism software version 5.01 (GraphPad Software, San Diego, CA). For pharmacological analyses, individual response curves were analyzed using the GraphPad software, and a mean curve was fit to the family of curves. Two-way analysis of variance with Neumann–Keuls post hoc testing was performed on relevant parameters from pharmacological and histological analyses to compare young and old STC animals. One-way analysis of variance (when AMC were used for comparison) and Student's *t*-test (when only STC animals were analyzed) were performed on cystometric evaluations and pharmacological experiments if only one age group was evaluated (ie, old animals at 2, 4, and 8 weeks after STC), with Neumann–Keuls post hoc testing used to compare groups. *P* values of <0.05 were considered significant in all cases. Except as otherwise stated, data are expressed as means \pm SEM.

Results

Subtotal Cystectomy

Of the 38 young animals (12 weeks old) that underwent STC, 4 died within 3 days because of urine leakage into the

peritoneum (11% mortality). When STC was performed in 82 old animals (12 months old), 18 animals died, and five more were euthanized because of complications such as stones or abscesses (28% morbidity and mortality). Young and old animals that survived STC gained body weight similarly to AMC animals at all time points after STC. At 6 months after STC, old animals weighed significantly more than young animals; however there was no change in body weight due to STC (223.0 ± 5.0 g young control versus 230.3 ± 3.3 g young STC; 286.9 ± 7.9 g old control versus 289 ± 11.7 g old STC).

At 8 weeks after STC, mean bladder weights were significantly lower in old STC animals (99.6 ± 8.5 mg, 87.3 ± 10.3 mg, and 122.2 ± 7.8 mg at 2, 4, and 8 weeks, respectively) than in AMCs (160.7 ± 7.2 mg, 142.7 ± 10.4 mg, 169.7 ± 10.2 mg for 2, 4, and 8 weeks). At 6 months after STC, however, no differences were found in bladder weight due to age or STC; bladders from young animals weighed 121.0 ± 9.2 mg (control) and 115.7 ± 6.9 mg (STC), and those from old animals weighed 141.9 ± 9.8 mg (control) and 124.6 ± 5.4 mg (STC).

Increased kidney size and ureteral dilation were observed in old animals subjected to STC, with significantly higher average weight of both kidneys from STC animals at 6 months after STC (Figure 1A). Of the 16 old animals that survived 6 months after STC, 8 showed signs of unilateral kidney damage, 3 had bilateral kidney damage, and 5 had no detectable kidney damage. None of the young animals had kidney damage at 6 months after STC. The observed increase in kidney weight reached significance, even though there was often a large difference between kidneys from the same animal due to compensation. For example, STC in one old animal resulted in two kidneys weighing 0.45 g and 1.25 g. This compensation was also evident in the variability of creatinine clearance, with only two old STC animals exhibiting impaired kidney function (Figure 1B). Histological analysis revealed typical hallmarks of kidney damage, such as hyaline casting,

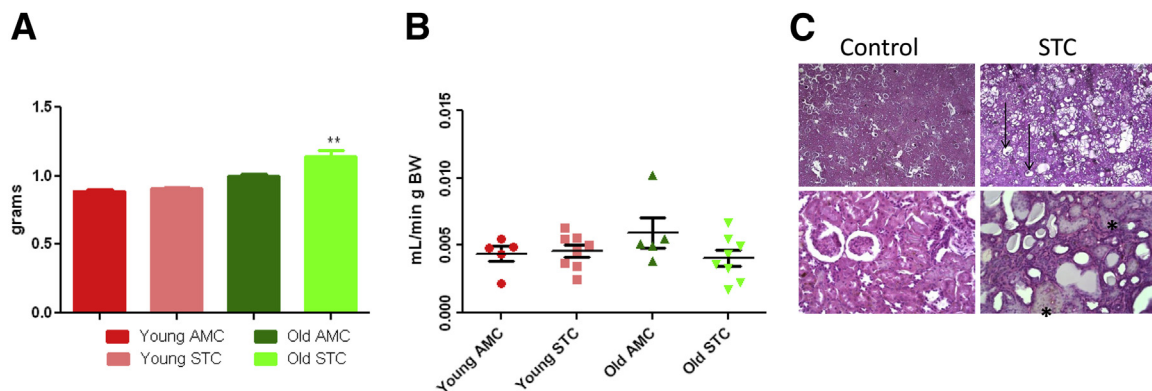


Figure 1 Effects of STC on kidneys. **A:** Average weight of both kidneys increased significantly in old STC animals at 6 months after STC, compared with the other three groups. **B:** Creatinine clearance, a measure of kidney function, did not differ between groups at 6 months after STC. **C:** H&E staining reveals kidney damage in an old animal at 6 months (26 weeks) after STC. Damage in the form of hyaline casts (asterisks) and increased intracapsular space (arrows) is present in old animals after STC, but not in control animals. Data are expressed as means \pm SEM; individual data points in **B** illustrate variability within groups. $^{**}P < 0.01$. Original magnification: $\times 50$ (**C**, top); $\times 400$ (**C**, bottom). AMC, age matched control; BW, body weight.

Table 1 Urodynamic Parameter Estimates for Old and Young Control and STC Animals Determined by *in Vivo* Cystometry at Different Time Points

Group and time point	Sample size	BC (mL)	MV (mL)	RV (mL)	BP (cmH ₂ O)	TP (cmH ₂ O)	MP (cmH ₂ O)	B _{com} (mL/cmH ₂ O)
Old control*	n = 8	0.88 ± 0.05	0.88 ± 0.07 [†]	0.04 ± 0.02	20.36 ± 3.45	31.74 ± 3.84	55.44 ± 5.18	0.08 ± 0.01
Old STC, 2 weeks	n = 9	0.37 ± 0.04 [†]	0.35 ± 0.04 [†]	0.03 ± 0.01	9.18 ± 1.28 [†]	16.98 ± 1.15 [†]	22.96 ± 1.86 [†]	0.05 ± 0.00 [†]
Old STC, 4 weeks	n = 9	0.47 ± 0.02 [†]	0.44 ± 0.03 [†]	0.03 ± 0.008	10.78 ± 1.68 [†]	19.19 ± 3.01 [†]	30.71 ± 3.71 [†]	0.07 ± 0.01
Old STC, 8 weeks	n = 9	0.54 ± 0.06 [†]	0.51 ± 0.08 [†]	0.05 ± 0.01	11.75 ± 1.49	20.53 ± 2.71	33.28 ± 4.21 [†]	0.07 ± 0.01
Old control, 6 months	n = 7	0.84 ± 0.13	0.74 ± 0.12	0.1 ± 0.02	16.45 ± 2.50	23.69 ± 2.80	55.14 ± 5.32	0.13 ± 0.03
Old STC, 6 months	n = 7	0.47 ± 0.06 [†]	0.39 ± 0.05 [†]	0.08 ± 0.01	12.34 ± 6.29	21.36 ± 5.03	32.08 ± 2.23 [†]	0.09 ± 0.02
Young control, 6 months	n = 5	0.78 ± 0.09	0.73 ± 0.07	0.06 ± 0.02	13.98 ± 1.74	23.49 ± 2.32	60.19 ± 5.37	0.13 ± 0.04
Young STC, 6 months	n = 8	0.75 ± 0.06	0.70 ± 0.06	0.05 ± 0.02	11.40 ± 1.61	19.19 ± 2.39	34.42 ± 4.44 [†]	0.10 ± 0.01

Data are expressed as means ± SEM.

*Age matched controls for old animals at 2, 4, and 8 weeks after STC revealed no differences and thus were pooled as controls.

[†]P < 0.05 versus corresponding control animal.

BC, bladder capacity; B_{com}, bladder compliance; BP, basal pressure; MP, maximal pressure; MV, micturition volume; RV, residual volume; TP, threshold pressure.

tubular degeneration, and increased intracapsular space (Figure 1C).

Cystometric Analysis

Values for cystometric parameters from all STC and age-matched control animals are presented in Table 1. At no time after STC did bladder capacity in the old animals return to the corresponding AMC values. In fact, at the 6-month time point, the bladder capacity of the old STC animals was still only slightly more than one half (approximately 56%) of that observed in AMC animals. Moreover, the ratio of residual to micturition volume (RV/MV) increased linearly over time in the old STC animals (Figure 2), but not in the young STC animals. By 6 months after STC, there was a clear and statistically significant difference in the RV/MV ratio, consistent with an impaired ability of the bladder from old STC animals to empty. In contrast, and consistent with previous findings at 8 weeks after STC,¹⁰ the bladder capacity of the young animals at 6 months after STC was indistinguishable from that observed in AMC animals. Moreover, as noted above, there was no detectable alteration in the ability of regenerated bladders from young STC animals to empty at any time point after STC.

Basal pressure, threshold pressure, and maximal pressure were all significantly lower in the old STC animals at 2 and 4 weeks after STC (Table 1). By 8 weeks after STC, however, only maximal pressure remained significantly lower in the old animals, compared with the AMC levels, and was still significantly lower at the 6-month time point. Interestingly, although both young and old STC animals exhibited similar reductions in maximal pressure after STC at the 6-month time point, the RV/MV ratio was increased only in the old STC animals. Finally, bladder compliance significantly decreased in old animals 2 weeks after STC,

but had returned to normal values by 4 weeks after STC. Overall, these data indicate a decline in function of the regenerated bladder in the old but not the young animals.

In Vitro Studies

Carbachol-Induced Contractions in Regenerating Bladders at 2, 4, and 8 Weeks after STC

To further evaluate the characteristics of the regenerated bladder, pharmacological studies were conducted on bladder strips from all treatment groups. Steady-state CRCs were obtained for carbachol-induced contractile responses in bladder strips from both AMC and STC animals. Mean values for logistic parameter estimates for carbachol are presented in Table 2. In short, logistic analysis revealed a significant reduction in the calculated maximal steady-state

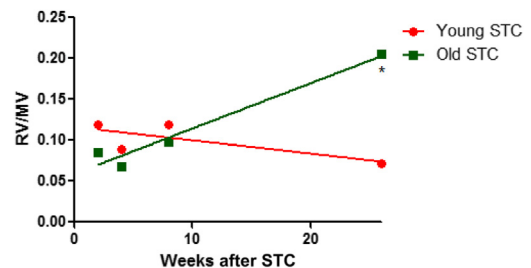


Figure 2 Linear regression over time of the RV/MV ratio in young and old animals after STC. Mean values for this calculated parameter for young animals at 2, 4, and 8 weeks are according to Burmeister et al 2010.¹⁰ At 6 months (26 weeks) after STC, old animals were unable to completely empty the bladder, as indicated by the high RV/MV ratio. Linear regression of the RV/MV ratio in old animals after STC ($y = 0.0055x + 0.06$, $r^2 = 0.96$, $P = 0.0196$) revealed an increase in RV/MV, but revealed no change in the ratio in young animals after STC ($y = 0.0016x + 0.12$, $r^2 = 0.58$, $P = 0.2373$). The RV/MV ratio was significantly higher in old animals than in young animals at 26 weeks after STC ($P = 0.0334$, Student's *t* test). * $P < 0.05$.

Table 2 Logistic Parameter Estimates Obtained from Carbachol CRCs on Old Animals at 2, 4, and 8 Weeks after STC

Group and time point	Animals (strips)	E_{\max}	pEC ₅₀	Hill slope
Old control*	$n = 8$ (18)	384.1 ± 16.1	6.01 ± 0.08	1.21 ± 0.25
Old STC, 2 weeks	$n = 7$ (13)	$127.0 \pm 19.0^{\dagger}$	5.76 ± 0.28	0.90 ± 0.48
Old STC, 4 weeks	$n = 9$ (13)	$64.3 \pm 6.9^{\dagger}$	5.90 ± 0.21	1.17 ± 0.59
Old STC, 8 weeks	$n = 9$ (15)	$76.8 \pm 7.1^{\dagger}$	5.74 ± 0.30	0.87 ± 0.48

*Age matched controls for old animals at 2, 4, and 8 weeks after STC revealed no differences and thus were pooled as controls.
 $^{\dagger}P < 0.05$ versus control.
 E_{\max} , calculated maximal steady state response; pEC₅₀, the negative log of the agonist concentration that results in 50% of the maximum response.

response (E_{\max}) values in isolated detrusor strips from the regenerating bladder at all time points after STC. There were no detectable differences in either pEC₅₀ or Hill slope parameters among the groups (Table 2).

Comparison of Contractility in Bladder Strips from Old and Young Rats at 6 Months after STC

To gain insight into the long-term physiological consequences of regeneration, detailed studies were performed on strips from bladders at the 6-month time point after STC. KCl-induced contractions of bladder strips revealed that maximal contractions in tissue from young STC animals were lower than those observed in bladder strips obtained in AMC animals, but higher than in old STC animals (Figure 3A). In addition, steady-state CRCs were obtained for carbachol- and phenylephrine-induced contractile responses in bladder strips from both AMC and STC animals at 6 months. Mean values for logistic parameter estimates are presented in Table 3. The calculated E_{\max} values for carbachol CRCs were lower in bladder strips from young STC animals at 6 months after STC, compared with AMCs, but higher than in bladder strips from old STC animals; however, the calculated E_{\max} values for bladder strips from young and old AMC rats did not differ (Table 3 and Figure 3B). No detectable differences were observed for either pEC₅₀ or Hill slope parameters among the groups (Table 3). Phenylephrine-induced cumulative steady-state CRCs also revealed a lower calculated E_{\max} value in bladder strips from old STC animals, compared with all other groups; again, there were no significant differences in the pEC₅₀ or Hill slope values among the groups (Table 3 and Figure 3C).

The maximum contractions observed in detrusor smooth muscle strips from regenerating bladders induced by electrical field stimulation 6 months after STC are presented in Figure 3D. Responses of bladder strips from both old and young STC animals were significantly lower than their AMC groups at all frequencies tested. Moreover, the STC/control ratio of the electrical field stimulation induced contractile responses of isolated bladder strips was similar across all frequencies (Figure 3D).

Composition of the Bladder Wall after STC

Trichrome staining revealed that the wall of regenerating bladders contained urothelial, lamina propria, and smooth muscle layers in both age groups at 1, 2, 4, 8, and 26 weeks

after STC (Figure 4, A and B). Images were quantified for smooth muscle and collagen content of the bladder wall (Figure 4C). Two-way analysis of variance revealed a significant effect of age ($P < 0.001$) and time point ($P < 0.001$), as well as an age \times time point interaction effect ($P < 0.001$). Of note, significantly lower smooth muscle content was detected in old than in young STC animals.

Cellular Proliferation in Regenerating Bladders after STC

An initial evaluation of the extent of cell proliferation at 1, 2, 4, 8, and 26 weeks after STC was explored via immunostaining against proliferating cell nuclear antigen (PCNA) (Figure 5). Staining in tissue from control bladders always revealed a very low level of proliferating cells ($< 1\%$; data not

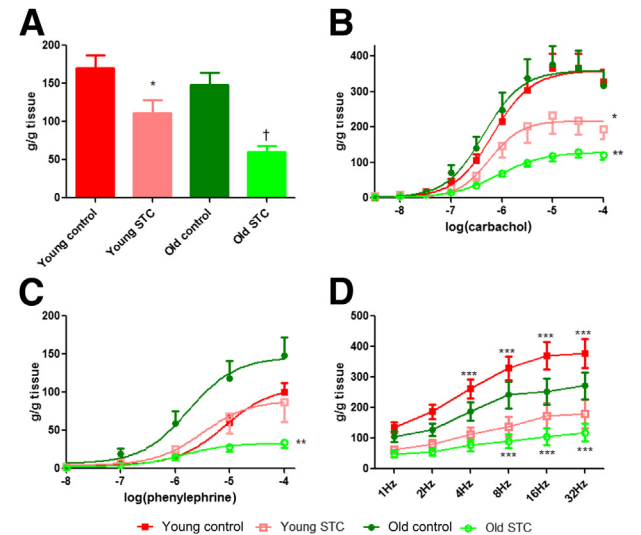


Figure 3 Pharmacological responses of bladder strips excised from rats 6 months after STC, compared with age matched control animals. **A:** Depolarization with KCl revealed significantly lower contractile responses in both young and old STC animals, compared with their controls. **B:** Carbachol CRCs revealed lower maximal responses in STC animals than in their controls, and also lower maximal responses in old than in young STC animals. **C:** Phenylephrine CRCs revealed a lower maximal response in old STC animals. **D:** Baseline frequency responses normalized to strip weight revealed lower contractile responses in STC animals than in their controls. Young control: 7 animals, 14 strips; young STC: 8 animals, 13 strips; old control: 6 animals, 12 strips; old STC, 9 animals, 14 strips. Data are expressed as means \pm SEM. * $P < 0.05$, ** $P < 0.01$ versus corresponding age matched control; $^{\dagger}P < 0.05$ versus all other groups.

Table 3 Logistic Parameter Estimates for Carbachol and Phenylephrine CRCs on Old and Young Animals at 6 Months after STC

Group	Animals (strips)	Carbachol			Phenylephrine		
		E_{\max}	pEC ₅₀	Hill slope	E_{\max}	pEC ₅₀	Hill slope
Old control	<i>n</i> = 6 (12)	358.4 ± 22.8	6.36 ± 0.13	1.15 ± 0.33	160.9 ± 38.2	5.70 ± 0.43	0.64 ± 0.44
Old STC	<i>n</i> = 10 (19)	128.3 ± 7.90**	6.08 ± 0.12	0.95 ± 0.21	35.6 ± 8.8**	5.84 ± 0.48	0.68 ± 0.56
Young control	<i>n</i> = 7 (14)	357.7 ± 18.1	6.18 ± 0.10	1.13 ± 0.24	113.0 ± 21.5	5.05 ± 0.27	0.83 ± 0.38
Young STC	<i>n</i> = 11 (17)	216.3 ± 17.3*	6.25 ± 0.15	1.38 ± 0.55	90.34 ± 23.8	5.50 ± 0.50	0.93 ± 0.81

Calculated logistic parameter estimates from CRCs generated from stimulation of bladder strips to carbachol and phenylephrine. The calculated maximal steady state response (E_{\max}) to cholinergic stimulation was lower in STC animals compared to age matched controls in either age group. Only bladders from old STC animals revealed a reduced E_{\max} when subjected to adrenergic stimulation.

* $P < 0.05$.

** $P < 0.01$ versus corresponding control.

shown). In STC animals, PCNA immunostaining revealed a significantly higher level of positively stained cells in the bladder wall in young animals than in old animals at 1, 2, and 4 weeks after STC. Consistent with this observation, quantification of PCNA staining and subsequent statistical analysis (two-way analysis of variance) revealed diminished cellular proliferation in the bladder wall of old STC animals from 1 to 4 weeks after STC, compared with young STC animals; this trend was reversed at 8 weeks, when an increase in the number of PCNA-positive cells was actually greater in the old animals than the young animals. There were no differences in the amount of PCNA immunostaining between proximal and distal sections (data not shown).

Angiogenesis in Regenerating Bladders after STC

We also examined potential age-related differences in angiogenesis at 1, 2, 4, 8, and 26 weeks after STC, by staining the vasculature of the bladder wall for VWF. Representative examples of VWF staining in bladder wall from young and old animals are shown in Figure 6, A and B. Quantification of VWF staining revealed similar average baseline (control) vessel circumference measurements of $89.1 \pm 7.3 \mu\text{m}$ and $86.7 \pm 2.5 \mu\text{m}$ in the bladder wall of young and old animals, respectively; moreover, statistical analysis revealed no detectable effect of age or time point on vessel circumference, and no age × time point interaction (Figure 6C). At 1 and 2 weeks after STC, however, there were significant differences in the number of blood vessels in the bladder wall of young animals (13.9 ± 2.8 and 31.9 ± 2.2 vessels per $20\times$ field of view at 1 and 2 weeks, respectively) versus old animals (6.6 ± 1.4 and 14.4 ± 2.2 vessels per $20\times$ field of view, respectively) (Figure 6D).

Discussion

Numerous chronic bladder conditions adversely affect patient quality of life, and can threaten the upper urinary tract causing end-stage renal disease. Current surgical interventions are not always effective and can have adverse effects.^{16–19} Animal studies using a wide variety of synthetic and natural grafts (both cell seeded and unseeded) have documented a reasonable amount of mesenchymal and stromal incorporation after implantation. In particular, one such combination of a collagen–polyglycolic acid scaffold with dual seeded urothelial and smooth muscle cells has had moderate success in young patients with congenital spinal cord disease.⁹ However, a much larger population of old patients with bladder dysfunction (and a generally reduced regenerative capacity) could benefit from improved regenerative medicine and tissue engineering approaches to bladder restoration, reconstruction, and replacement. The rapid increase in tools and technologies becoming available for modification of both cellular and graft materials (eg, micro- or nanoparticles for delivery of molecules such as growth factors or oxygen, among many others), leading to

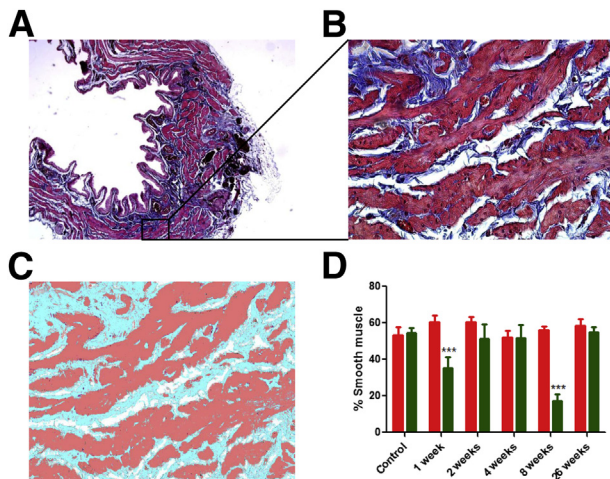


Figure 4 Quantification of muscle versus collagen content. **A:** Tri-chrome staining of native young bladder. The boxed region is shown at higher magnification in the adjacent panel. **B:** High power images (four per section) of the muscle layer were used for quantitative analysis. **C:** The same image, quantified for percent muscle content, with total muscle pixels as pink and collagen pixels as blue. **D:** Quantification of trichrome analysis used three different sections from at least three different animals in controls [native tissue excised from young (red bars) and old (green bars) animals during STC] and in animals at different time points after STC. Two way analysis of variance revealed a significant effect of age ($P < 0.0001$) and time ($P < 0.0001$), and a significant interaction effect ($P < 0.0001$). Post hoc analysis revealed a lower smooth muscle content in old STC animals, compared with young STC animals. *** $P < 0.001$, post hoc analysis. Original magnification: $\times 50$ (A); $\times 400$ (B).

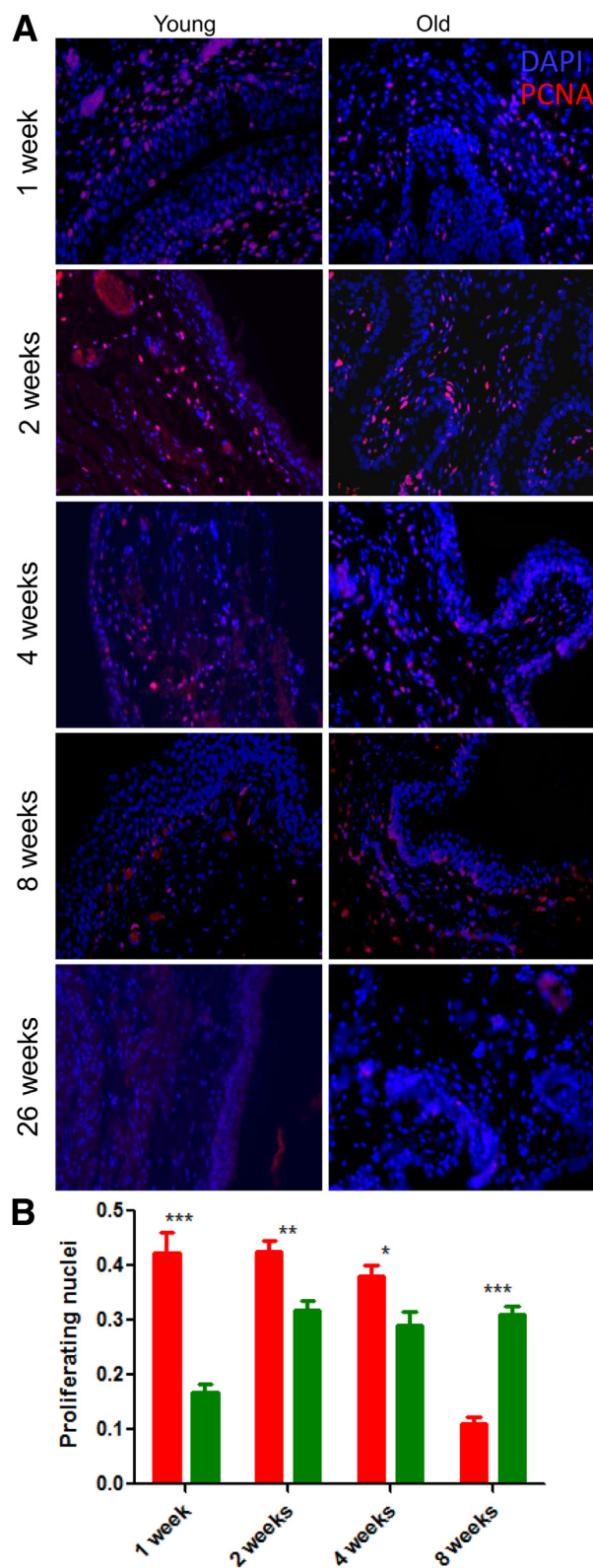


Figure 5 Cell proliferation in young and old STC animals. **A:** Representative images of PCNA staining from young and old animals at five time points, up to 26 weeks after STC. **B:** Semiquantitation of immunohistochemical PCNA staining revealed fewer proliferating cells present in bladders of old (green bars) animals at 1, 2, and 4 weeks after STC, but more proliferating cells at 8 weeks after STC, compared with young (red bars) animals at the same time points. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Original magnification, $\times 400$.

a more rigorous understanding of cellular and molecular mechanisms of native bladder regeneration, should greatly aid in the identification of targetable pathways to harness and augment natural regenerative abilities.

The importance of understanding normal bladder regenerative processes and capabilities is a key point, because the mechanisms responsible for endogenous regeneration of bladder tissue cannot be assumed to be identical to those invoked after the implantation or addition of cells or biomaterials. In fact, it corresponds to intuition that characterizing the native regenerative process would provide the best blueprint for development of improved regenerative therapies for bladder disease and dysfunction.

As a first step in this direction, we have used a rodent model of subtotal cystectomy to examine time-dependent changes in function and morphology of the regenerating mammalian bladder. The lower urinary tracts of rat and human have many similarities, and both have significant regenerative potential. Of note, the difference in age groups chosen for the present study is moderate. Well-documented survival curves of Fisher F344 female rats show that young (12 weeks old) and old (12 months old) animals achieve nearly 100% survival.²⁰ Although both age groups represent sexually mature phenotypes (ie, adults), 12-month-old rats are more accurately classified as middle-aged and typically do not present with issues in baseline bladder function.^{21,22}

However, despite the modest difference in age, our old animals subjected to STC exhibited increased morbidity and mortality (28%), compared with young animals (11%). The cause of death was similar in both age groups (namely, urine leakage into the peritoneum), and the death usually occurred

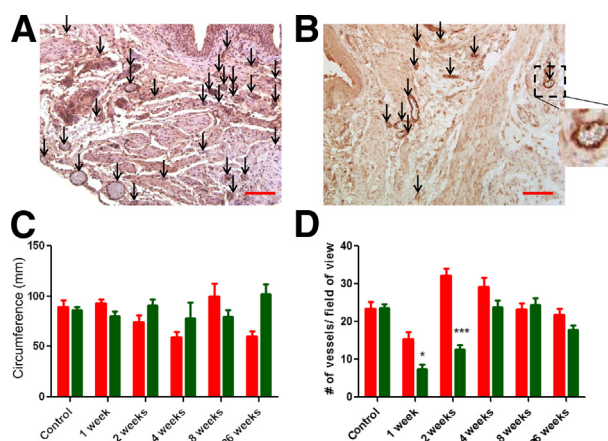


Figure 6 Quantification of vessel size and number. **A** and **B:** Representative images with immunostaining against VWF show blood vessels (arrows) in the bladder wall at 2 weeks after STC in young (**A**) and old (**B**) animals. **C** and **D:** Although no differences were found in terms of blood vessel wall circumference (**C**), a significant difference in the total number of vessels per high power field of view was found (**D**). Two way analysis of variance revealed a significant effect of age ($P < 0.0001$) and time ($P < 0.0001$), and a significant interaction effect ($P < 0.0001$). Post hoc analysis revealed a lower amount of vessels after STC in old animals (green bars) compared with young animals (red bars). * $P < 0.05$, *** $P < 0.001$ post hoc analysis. Original magnification, $\times 200$. Scale bar = 100 μm .

within 3 days after STC. This observation is consistent with an impaired ability of the urothelium and the bladder wall to reseal immediately after STC in old animals. This supposition is consistent with the decreased PCNA staining observed at 1 and 2 weeks after STC in some of the old animals (Figure 5). Interestingly, the age-related difference in PCNA expression appears to be largely due to an increase in cellular proliferation in the lamina propria layer (data not shown). In this regard, the normally quiescent urothelium rapidly proliferates in response to injury.²³ Although this response has been suggested to be essential in regeneration of the bladder, in the present study we did not explore the effect of age on urothelial proliferation in any depth, and the question remains open for future investigation.

The most serious adverse effect due to STC in the surviving old animals was kidney damage. Enlarged, edematous kidneys developed, and histology revealed chronic kidney damage in the form of hyaline casts and tubular degeneration (Figure 1). Kidney enlargement was usually unilateral, with sufficient compensation by the contralateral kidney, as is also seen clinically.²⁴ This functional compensation was further evidenced by normal average creatinine clearance in old animals at 6 months after STC. The exact reason for damage to the kidneys is unclear. Hydronephrosis due to high intravesical pressures may be one explanation for urine reflux; however, urodynamic investigation did not reveal increased basal pressures. Obstruction of the ureteral orifice into the bladder could be a feasible explanation, but such obstruction was not obvious upon animal sacrifice. Natural repair mechanisms of the kidney in response to injury (eg, ischemia reperfusion) are known to be impaired with aging, and several mechanisms have been reported to be involved, including reduced epithelial proliferation, decreased stem cell or progenitor cell function, enhanced susceptibility to apoptosis, and telomere shortening.^{25,26} Although there is no evidence for implicating these processes in our model, any strain on the kidneys after STC may exhaust baseline repair mechanisms in old animals.

In several models of mammalian organ regeneration, regenerative capacity has been shown to decrease with age, and various possible explanations have been suggested. One recent study examined liver regeneration, in which 70% hepatectomy resulted in age-associated deficiencies in cell proliferation within 48 hours after resection of the liver.²⁷ This was reversed with a serotonin receptor agonist that worked through a VEGF-dependent pathway. Indeed, angiogenesis has been shown to be essential in many models of organ regeneration (as has been reviewed elsewhere^{28,29}). In our model, significant age-related changes in morphology were observed, with both cell proliferation (PCNA staining) and the number of newly formed vessels significantly lower in old animals at 1 and 2 weeks after STC. Thus, the present findings also illustrate the importance of both cell proliferation and angiogenesis in organ regeneration.

Skeletal muscle is another organ with a substantial regenerative capacity in the adult mammal, and age-dependent mechanisms for diminished regenerative potential have been

reported, including reduced stem (satellite) cell function and fibrosis.^{30–34} We observed a transient increase in collagen content in the bladders of old rats subjected to STC, but by 6 months after STC the young and old animals showed similar ratios of smooth muscle to collagen, which suggests minimal overall effect of fibrosis in this model at these time points. The lack of a definitive bladder stem cell further complicates the examination of stem cell numbers or function.^{35,36} Studies using label-retaining methods suggest that the basal urothelial cell layer is the stem cell niche for the urothelium.³⁷ Although the precise location of cellular proliferation was not our main goal in the present study, consistent with our prior observations at 1 to 7 days after STC with bromodeoxyuridine labeling¹⁵ the PCNA staining reported here was also localized, although not exclusively, to basal cells. Also consistent with the earlier observations,¹⁵ a significant number of PCNA-positive cells were located in the lamina propria layer, and between (but usually not within) muscle bundles. This finding, coupled with our previous observation of an increase in c-Kit-positive cells in this same region,¹⁰ suggests a possible role for the myofibroblast/interstitial cell in regeneration. Although a role has been suggested for interstitial cells and innervation,³⁸ overall the mechanisms and origin of *de novo* smooth muscle formation remain unclear.

Inflammation is another aspect of tissue regeneration that has been shown to negatively affect function or regeneration of, for example, skeletal muscle and the prostate.^{39,40} Although time-dependent changes in inflammatory leukocytes have been shown in a rat bladder augmentation model, it is not possible to directly compare our results with that model, because it uses a xenograft.⁴¹ Additionally, it has been shown that M1 and M2 macrophages elicit differences in repair, and can be profibrotic (M1) or proregenerative (M2).⁴² Examining the age-related differences in cells and cytokines mediating inflammation may identify targets for altering the process of bladder regeneration, but the precise role of the inflammatory cascade remains to be elucidated.

Because the success of organ regeneration is measured largely by the degree of functional restoration,⁴³ in the present study we examined the effect of age on bladder function after STC. In this regard, the bladder serves as a high-capacity reservoir at low intravesical pressures during the storage phase, until micturition, which normally empties the bladder completely. In our urodynamic studies, bladder capacity in the old animals did not return to normal control values in 8 weeks after STC, a time frame shown previously to be sufficient for complete recovery of bladder capacity in young animals.¹⁰ Moreover, by 6 months after STC, bladder capacity in old animals was still only approximately 56% of AMCs, whereas capacity in young animals was equivalent to that of their controls. Although there was no significant age-related difference in residual volume, there was a significant decrease in micturition volume (due to decreased bladder capacity), which resulted in a statistically significant linear increase in the RV/MV ratio over time.

The greater than twofold increase in the RV/MV ratio in the old animals at 6 months after STC (Figure 2) is a clear indication that bladder function is declining. Although this explanation is strictly speculative at present, the observed decline could certainly be due to the delayed proliferative (Figure 5) and angiogenic (Figure 6) responses observed histologically in old animals.

In all STC animals, regardless of age, there was a significant reduction in maximum pressures generated during the voiding cycle. Although the cause is unclear, possibilities include reduced outflow resistance in the urethral sphincter and/or a reduction in the overall strength of the detrusor smooth muscle contractions. The latter explanation is supported by a reduction in the maximal responses of bladder strips to the depolarizing agent KCl. Although these responses were reduced in young animals at 6 months after STC, contractility of bladder strips from old animals at this time point was even further compromised. Maximal steady-state contractile responses to carbachol exhibited a similar pattern between young (reduced response) and old (even more reduced response) animals after STC (Table 3), and Saito et al¹⁴ reported significant reductions in contractility after STC. Frederiksen et al,¹¹ however, reported no difference in contractility. The discrepancy between studies may be due to differences in strip preparations (ie, transverse versus longitudinal). Additionally, although other studies have reported a reduction in contractility due to age alone,⁴⁴ albeit in much older animals (28 to 30 months old), we did not observe any effect of age on contractile responses, most likely because of the modest age differences between our young and old groups.

Because the depolarization-induced contractions in response to KCl are diminished to a similar degree as are the receptor-mediated contractions induced with carbachol, phenylephrine, and electrical field stimulation (Figure 3), it seems that the observed reductions in contractility in bladders from animals subjected to STC are not membrane receptor-mediated, but rather that the putative mechanism or mechanisms arise downstream of receptor activation. In this regard, Frederiksen et al⁴⁵ showed that there are no differences in the total amount of smooth muscle actin, and that the ratio of actin to myosin remains unchanged, at 15 weeks after cystectomy. However, changes in cross-bridge efficiency and kinetics may be explained by changes in smooth muscle myosin isoforms of either the light or heavy chain. For example, DiSanto et al⁴⁶ showed that the smooth muscle myosin heavy chain isoform SM-A has a decreased maximum velocity and shortening length, and that a change to this isoform may explain dysfunctional bladders after partial outlet obstruction.

In conclusion, the present study demonstrates that even modest aging in this study, the group defined as old was only in middle age (12 months) has a measurable adverse effect on the regenerative capacity of the bladder after STC. This fact is reflected in the nearly 50% decrease in bladder capacity (Table 1) and the greater than twofold increase in the RV/MV ratio (Table 1 and Figure 2) observed 6 months

after STC in the old compared with young animals. These functional changes in the bladder were associated with significant damage to the kidney in the old animals, which provides further indication of the potential clinical utility of this model. Moreover, diminished regenerative capacity was accompanied by significant reductions in cellular proliferation and angiogenesis in the bladder wall during the first 2 weeks after STC. The precise cellular and molecular mechanisms responsible for this age-related decline in functional regeneration of the bladder remain to be determined.

In addition, the present findings suggest that even greater reductions in regenerative potential might be expected even in old animals. These studies confirm and extend prior work and suggest that the rat can be a valuable model for studying age-related impairments in mammalian organ regeneration. Future investigations in the rodent model should provide important mechanistic insights of value to the development of novel therapeutics for the treatment of diverse bladder diseases and disorders.

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